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(71) Applicant: CROP GENETICS INTERNATIONAL CORPORATION [US/US]; 7170 Standard Drive, Hanover, MD 21076 (US).

(71)(72) Applicants and Inventors: FAHEY, Jed, W. [US/US]; 7512 Sea Change, Columbia, MD 21045 (US). HAKIMI, Mohammad, S. [US/US]; 5915 #3, Tamar Drive, Columbia, MD 21045 (US).

(74) Agents: PATTERSON, Herbert, W. et al.; Finnegan, Henderson, Farabow, Garrett & Dunner, 1300 I Street, N.W., Washington, DC 20005-3315 (US).

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(54) Title: METHOD OF ENDOPHYTE-ENHANCED PROTECTION OF PLANTS

(57) Abstract

A method of endophyte-enhanced protection in commercially-valuable plants is described which comprises providing an endophytic bacterium that can be harbored within the plant but creates no visible manifestation of disease and introducing that organism to the plant to enhance protection against a broad spectrum of diseases.

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METHOD OF ENDOPHYTE-ENHANCED PROTECTION OF PLANTS

FIELD OF THE INVENTION

The present invention relates to a method of providing enhanced protection against disease in commercially-valuable plants. More particularly, the present invention relates to such a method employing endophytic microorganisms.

BACKGROUND OF THE INVENTION

Since the first development of agriculture, man has battled to protect valuable plants from attack by bacteria, viruses, fungi, and insect pests that can rob him of the product of his labor and, on occasion, even threaten his existence. The focus in the past has been on chemical means of protection. Recently, the increased awareness of the effects of chemicals on the environment has led to the search for other, less toxic means of protecting plants. Mechanisms for biological control may provide a solution to this problem; however, to date they have proven to be largely ineffective.

Biological control of plant pathogens can be defined as "the decrease of inoculum or the disease-producing activity of a pathogen accomplished through one or more organisms, including the host plant but excluding man." See, K.F. Baker, Annual Review of Phytopathology 28: 67-85 (1987). The term was first used in relation to plant pathogens in 1914 and to insects in 1919.

One form of biological control is the phenomenon of induced resistance, that is, an increase in a plant's ability to resist disease after prior exposure to a pathogen. Although the mechanism of action of induced resistance (also called cross protection, acquired resistance or acquired immunity) has never been fully understood, it has been reported in the scientific literature since the 1950's. C.W. Bennett described virus infection to protect plants by induced resistance in Advances in Virus Research 1:39 (1953). Other early

reports on induced resistance include T.O. Biener (Annual Review of Phytopathology 1:197 (1963)) and B. Kassanis (Advances in Virus Research 10:219 (1963)).

These early reports of induced resistance described resistance that was conferred to plants by the introduction of biotic inducers, i.e., pathogenic inducers that were either live or attenuated (i.e., unable to live and increase within the plant). Although these inducers may have created the desired response in the plant, target crops and non-target species were subjected to pathogens that could be potentially harmful to them. In addition, these pathogens were often applied topically thus enhancing the opportunities for environmentally mediated inactivation of the organism (e.g. UV degradation). Moreover, topical application required relatively large amounts of the pathogen, enhancing the opportunity for unwanted exposure of non-target species. In addition when attenuated pathogens were used, multiple applications were often required.

In addition to the biotic inducers described above, abiotic (i.e., biochemical) inducers have also been reported in the scientific literature, (Modderman, P.W., et al., Phytopath. Zeit. 113:165-170 (1985); Albersheim, P.A., et al., Structure and Function of Plant Genomes, NATO Adv. Study Inst. Series. Plenum Publ. Corp. N.Y. pp. 293-312 (O. Ciferri (ed.) 1982); Graham, T.L., et al., Applied & Environmental Microbiology 34:424-432 (1977); van Loon, L.C., Netherlands Journal of Plant Pathology 89:265-273 (1983); Soliman, H.N., Egyptian Journal of Phytopathology 18(1):35-45 (1986); Salt, S.D., et al., Physiological & Molecular Plant Pathology 28(2):287-297 (1986); White, R.F., Virology 99:410-412 (1979); Gianinazzi, S. & B. Kassanis, Journal of General Virology 23:1-9 (1974); and van Loon, L.C., Virology 80:417-420 (1977)). These

inducers included such materials as chitin, oligosaccharides and polysaccharides, or other components of cell walls, in addition to chemicals such as salicylic acid.

Although those abiotic inducers were advantageous in that they were not likely to cause disease, there were numerous disadvantages inherent in the use of abiotic inducers including the ability to induce resistance only against a very limited spectrum of pathogens and the need for multiple applications. For example, application of oligosaccharides would induce resistance only against organisms with cell walls that were structurally similar to the inducing compound used. In addition, certain abiotics (e.g. $HgCl_2$) were potentially hazardous to work with and the "cure" caused damage to the plant which was worse than the disease. Other abiotics (e.g. UV light) were impractical to apply.

Accordingly, there remains a need for biological control of plant pathogens. Specifically, there is a need for a method for enhancing the natural mechanisms for resisting diseases present in commercially-valuable plants using an organism that will not harm the environment, will not harm the plant, is capable of living within the plant, requires only a single application, and will enhance protection against a broad spectrum of disease organisms.

SUMMARY OF THE INVENTION

The present invention overcomes the problems and disadvantages of the prior art by providing of method of enhancing disease resistance in commercially-valuable plants, comprising providing an endophytic organism which is capable of being harbored within the plant and which creates no visible manifestations of disease and, in one embodiment, creates no ill effects on the host plant. This organism is introduced into the plants to enhance protection against a wide spectrum of diseases. In addition, the present invention provides a method of

enhancing protection in commercially-valuable plants using a vascular-inhabiting endophyte, i.e., one that lives in the vascular tissues of the plant. In another embodiment, the invention relates to a method of enhancing protection using a vascular-inhabiting endophyte that is a gram positive bacterium. In still another embodiment, the present invention relates to a method of enhancing protection in commercially-valuable plants using an endophytic organism that lives in the vascular-inhabiting system of the plant, is gram positive, and is fastidious. The invention also provides for a method of enhancing protection in commercially-valuable plants using an endophytic organism known as Clavibacter xyli subsp. cynodontis (Cxc).

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be clear from the description, or may be learned by practice of the invention. These objects and advantages of the invention will be realized and obtained by means of the methods particularly pointed out in the appended claims.

It is to be understood that the general description above and the following detailed description and drawings are exemplary and explanatory only and do not limit the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several exemplary embodiments of the invention and, together with the description, serve to explain the principles of the invention.

Fig. 1 is a graph that depicts the effect of Cxc inoculation on leaf area of tobacco (variety C319) challenged fourteen days post-inoculation with tobacco mosaic virus (TMV).

Fig. 2 is a graph that depicts the effect of Cxc inoculation of leaf area of tobacco (variety C319) challenged twenty days post-inoculation with TMV.

Fig. 3 is a graph that depicts the effect of Cxc inoculation on leaf weight of tobacco (variety C319) challenged fourteen days post-inoculation with TMV.

Fig. 4 is a graph that depicts the effect of Cxc inoculation on leaf weight of tobacco (variety C319) challenged twenty days post-inoculation with TMV.

Fig. 5 is a graph that depicts the titer of Pseudomonas syringae pv. tabaci titer in Cxc-inoculated tobacco (variety Ky-14) leaves.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will not be made in detail to the currently preferred embodiments of the invention, examples of which are illustrated below and in the accompanying drawings.

As used herein, "endophyte-enhanced protection" is defined as the reduction of disease in plants resulting from the introduction of an endophyte into plants. The present invention is not limited by the manner in which the endophyte enhances protection of the plant against disease, nor, as discussed more fully below, by the method of its introduction into plants.

Unlike the induced resistance previously described by the prior art, the endophytes of the present invention do not act as pathogens in the host plant. The endophytes are organisms that are capable of being harbored within the plant but create no visible manifestations of disease and, in one embodiment, have no ill effects on the host plant.

The endophytic organisms of the present invention may also be referred to as organisms which are capable of entering into an endosymbiotic relationship with a plant host. The endosymbiotic relationship is one in which the organism actually exists within and may spread throughout all or a portion of the host plant, without causing any

significant adverse effect on the host plant. The endosymbiotic relationship of an endophyte with a host plant in the present invention is not limited by the nature of the relationship and may include mutualistic and commensalistic endophytic organisms.

The endophytes used in the method of the present invention are contained within the plant body. In a preferred embodiment, the endophytes are contained within the vascular system of the plant or, in an alternative embodiment, within the intercellular spaces of the plant.

In another embodiment, the vascular-inhabiting or intercellular-space-inhabiting endophytes are gram-positive. "Gram-positive" refers to a classification of microorganisms based on the components of the cell wall as that term is described by Davis et al. in Microbiology, 3rd ed., (1980), specifically incorporated herein by reference.

In still another embodiment of the invention, the gram-positive vascular-inhibiting endophytes are fastidious in nature. As used herein, the term "fastidious" refers to organisms having complicated nutritional requirements, as that term is defined by McCoy, R.E., in "Chronic and insidious disease: The fastidious vascular pathogens," Phytopathogenic Prokaryotes (Mount M.S. and Lacy, G.H., eds. 1982), specifically incorporated herein by reference.

In still another embodiment, the present invention relates to endophytes of the Coryneform family as that term is defined by M.J. Davis in Annual Review Phytopathology 24: 115-40 (1986), specifically incorporated herein by reference. In another embodiment the present invention relates to the genus Clavibacter. In a particularly preferred embodiment, the invention relates to the endophyte known as Clavibacter xyli subsp. cynodontis (hereinafter "Cxc"), as that term is defined by M.J. Davis et al. in International Journal of Systematic Bacteriology 34(2):107-117 (April 1984), specifically incorporated herein by reference.

The present invention contemplates the introduction of live endophytes capable of being harbored within the plant host. The endophytes of the present invention may multiply within the plant host but the present invention is not limited to endophytes that multiply within the host.

The endophytes of the present invention may be unmodified or modified or formulated with other components to provide beneficial properties in addition to enhanced protection. Modification of endophytes is accomplished by techniques that are known to those of ordinary skill in the art. Any means of modification and any modification of endophytes are specifically contemplated by the present invention.

The endophytes used in the method of the present invention may be modified, for example, by mutagenesis or recombinant techniques known to those of ordinary skill in the microbiology and molecular biology art in light of the teachings contained herein. The endophyte may be modified by the induction and isolation of mutant strains effective in protecting plants against disease. The DNA of the endophytes may be modified by the addition of DNA that codes for the production of particular compounds, including but not limited to proteins, antibiotics, and other biochemical compounds. Thus, the endophyte could, in addition to enhancing protection, provide agricultural chemicals that might benefit the plant. On such method for the production of such endophytes is provided copending in United States Patent Application No. 166,819 (filed March 3, 1988), No. 266,232 (filed October 10, 1988), and No. 266,221 filed October 10, 1988), all of which are commonly assigned to the assignee of the present invention and are incorporated specifically herein by reference.

Alternatively, endophytes may be modified by mutagenesis or recombinant techniques to produce inducer compounds, such as, for example, dihydroxy benzoic acid or beta-ionone. The techniques for these modifications are

similarly within the routine skill of one ordinary skill in the art in light of the teachings contained herein.

In addition, plant protection provided by the present invention may be enhanced by formulating the endophyte with one or more abiotic inducers. The techniques to select abiotic inducers and develop formulations including them are within the routine skill of those of ordinary skill in the art in light of the teachings contained herein.

The modified, unmodified or formulated endophytes may be introduced to the plants by any technique known to those ordinary skill in the art. The method of endophyte introduction does not in any way limit the present invention. Introduction techniques, which vary with the plant host, include, but are not limited to, latex plugs, slow releases, root drips for transplanted plants, abrasive sprays, needle or needless injection, pressure injection and the like.

In a preferred embodiment, the endophytes are introduced by stem stabbing. "Stem stabbing" refers to the introduction of endophytes by wounding the plant and delivering the endophytes to that wound. A preferred method of stem stabbing involves a scalpel or other sharp instrument that is first coated with an endophyte and then used to simultaneously wound and deliver the organism.

In another embodiment, the endophytes are introduced to plants by stem injection. "Stem injection" refers to the introduction of organisms into the stem of the plant via a puncture created by a needle of, for example, a tuberculin intradermal syringe. In one preferred method, the needle of the syringe, containing the endophytic organisms to be introduced, is gently pushed into the stem and the contents of the syringe gently and slowly injected into the stem.

In another embodiment, the endophytes are introduced to plants either by injection into the petiole

by needle or by deposition onto a previously broken petiole.

In still another embodiment, the endophytes can be introduced by intercellular infiltration, where a suspension of endophytes is injected into the intercellular spaces of a leaf.

In still another embodiment of the invention, the endophytes may be introduced by inoculating the seeds of the plant with the endophytes. The method of seed inoculation is provided in co-pending United States Patent Application No. 194,247, filed May 16, 1988, to Jed W. Fahey, incorporated specifically herein in its entirety by reference.

The invention relates to enhanced protection in all commercially-valuable plants. Persons of ordinary skill in the art are generally familiar with agriculturally-valuable plants. These include the horticultural plants, such as those producing fruits, vegetables, flowers and ornamental trees and plants. In addition, commercially-valuable plants include agricultural trees and plants such as field and row plants. Field and row plants include, but are not limited to, corn, sorghum, wheat, barley, oats, rice, tomato, potato, cabbage, broccoli, melons, cucumbers and related plants. In another embodiment, commercially-valuable plants encompass plants of forestry. This list is exemplary only and does not in any way limit the application of the present invention.

In accordance with the present invention, the protected plants become resistant to one or more of a broad spectrum of diseases including, but not limited to, mildews, rusts, smuts, rots, scabs, spots, blights, blasts, decay, damping-off, leaf rolls, vascular wilts, warts, galls, yellows, cankers, mosaics, ring spots and other stunting, dwarfing or disfiguring plant diseases. These diseases include those caused by bacteria, viruses, and fungi and other biotic pathogens. In a preferred

embodiment, the plants are resistant to tobacco mosaic virus, potato viruses X and Y, Pseudomonas syringae pv. tabaci, Clavibacter michiganense subsp. michiganense and Fusarium oxysporum f.sp. melonis.

The invention will be further illustrated by the following examples, which are intended to be purely exemplary of the invention.

EXAMPLE 1

LOCAL LESION (HYPERSENSITIVE) RESPONSE IN ENDOPHYTE-INOCULATED TOBACCO PLANTS FOLLOWING CHALLENGE WITH TOBACCO MOSAIC VIRUS

Plants of Nicotiana tabacum L. cv. 'Ky 14', a variety hypersensitive to tobacco mosaic virus (hereinafter "TMV"), were planted in one gallon pots in the greenhouse. Thirty days after sowing, the plants were selected for uniformity. Plants were randomly assigned as either control plants or treatment plants.

A. Preparation of the Endophyte (Cxc):

For six days at 28°C ± 3°C, Cxc was grown on SC Media, consisting of 1000 ml distilled water; 17 g cornmeal agar; 8 g papaic digest of soy meal; 1 g K_2HPO_4; 1 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 15 mg (15 ml of a 0.1 percent solution in 0.05N NaOH) bovine hemin chloride; 2 g (10 ml of a 20 percent aqueous solution) bovine serum albumin fraction 5; 0.5 g (1.0 ml of a 50 percent aqueous solution) glucose; and 1 g (free base, 10 ml of a 10 percent aqueous solution) cysteine. After incubation, the cells were washed and suspended in 10 ml sterilized tap water. Suspensions of Cxc cells were centrifuged at 6000 rpm for 15 minutes and resuspended in sterile water or phosphate buffered saline (PBS). Bacterial concentration was determined spectrophotometrically at 600 nm and adjusted to ca. 10^8 bacteria/ml.

B. Inoculation with the Endophyte (Cxc):

The treatment plants were inoculated with solutions containing Cxc as follows:

1. - Stem Inoculations
   a) Injection of Cxc cells into the stem 4 cm above the soil surface by hypodermic syringe.
   b) Stabbing of Cxc into the stem by sterile scalpel blades containing Cxc scraped from streaked plates where the scalpel tip was inserted completely through the stem.
2. - Petiole Injections
   Injection of Cxc into the petiole by hypodermic syringe.
3. - Intercellular Infiltration.
   Injection of ca. 10 ul of bacterial suspension into the intercellular space of the leaf anima to create water soaking; eight injections per leaf, with hypodermic syringe.

In addition, control plants were inoculated with control solutions, i.e., the same solutions as above except that Cxc was absent.

C. Inoculation with the Challenge Organism:

Two weeks later, partially purified suspensions of strain U-1 of TMV in phosphate buffered saline were used for all challenge inoculations, as in R.W. Fulton, "Nicotiana As Experimental Virus Hosts, "Nicotiana Procedures for Experimental Use - Technical Bulletin No. 1586 (U.S.D.A. P.D. Durkin, ed., 1979), specifically incorporated herein by reference. A gauze pad was soaked in the TMV inoculum and rubbed onto all expanded leaves following a light dusting with 600 mesh carborundum, an abrasive powder, to facilitate viral infection.

As set forth in Table 1, prior inoculation of tobacco variety KY-14 with Cxc resulted in a consistent

reduction of TMV lesion numbers. Accordingly, prior inoculation with Cxc resulted in a dramatic reduction of the hypersensitive response compared to controls.

Table 1

Effect of Cxc Inoculation on Lesion Number
of Tobacco Variety Ky 14 CHallenged with TMV

Method of Inoculation	Mean # of lesions/100 sq cm leaf[a]			Percent Reduction In Lesions (Compared to uninoculated controls)		
	Rep#1	Rep#2	Rep#3	Rep#1	Rep#2	Rep#3
Stem Stab with Cxc	8	112	45	86	25	17
Stem Inject with Cxc	12	66	--	79	56	--
Petiole Inject with Cxc	12	--	--	79	--	--
Leaf Inject with Cxc	31	--	--	46	--	--
Uninoculated Control	57	150	54	0	0	0

a = Average of counts from 16 leaves
"-" = experiment not conducted

EXAMPLE 2

LOCAL LESION (HYPERSENSITIVE) RESPONSE IN ENDOPHYTE-INOCULATED TOBACCO PLANTS FOLLOWING CHALLENGE WITH TOBACCO MOSAIC VIRUS

In the same manner as Example 1, in a field experiment, Cxc-inoculated tobacco plants (variety KY 14), planted in a randomized complete block design with five replications, exhibited a reduction in lesion number over controls when plants were challenged at fourteen days and twenty days (two separate readings) post-inoculation (see Table 2 below). All plants (both control and experimental) challenged at thirty-one days showed such low numbers of lesions that comparisons between treatments is not valid, likely due to environmental conditions in the field.

Table 2

Effect of Cxc Inoculation on TMV Lesion Number in Field-Planted Tobacco Variety KY 14

	Method of Inoculation	Mean # of lesions/100 sq cm leaf			
		Days After Cxc Inoculation			
		14	20	20	31
Experimental	Stem Stab with Cxc	60	49	20	8.4
	Stem Inject with Cxc	60	41	14	6.9
Control	Stem Stab with Water	60	57	24	8.1
	Stem Inject with Water	63	52	23	8.7
	Unioculated Control	85	58	29	6.7
	LSD =	32.4	13.6	9.85	4.5
	N =	100	68	80	100

*LSD refers to Least Significant Difference

EXAMPLE 3

SYSTEMATIC RESPONSE IN ENDOPHYTE-INOCULATED TOBACCO PLANTS FOLLOWING CHALLENGE WITH TMV

Using the protocol of Example 1, sixty plants of tobacco variety Coker 319 (C319) were planted in the greenhouse. After five weeks of growth, uniform plants were selected and subjected to the following treatments:

1) untreated control
2) stem stab with water (control)
3) stem stab with Cxc
4) stem injection with Cxc.

The entire experiment was repeated three separate times.

Plants were inoculated with Cxc at the stage of growth when two true leaves had formed. Fourteen days later, the plants were challenged with TMV by inoculation of true leaves four and five (counted from the soil line). Twenty days later, leaves numbered 5, 6, 7, and 8, counted from the challenged leaf, were removed and leaf area and leaf fresh weight were assessed. Leaf fresh weight was determined by removing any adherent water or debris and weighing the entire leaf and subtending petiole. Nine days later, plants were re-assessed by measuring leaf area and fresh weight for leaves numbered 9, 10, 11, and 12. In addition, plant height was determined by measuring total height of the plant from the soil line to the uppermost leaves and plant weight was scored as the weight of the above-ground portion of the plant.

In two of the three replications, Cxc-inoculated plants exhibited greater leaf area and leaf weight than their respective controls. Table 3 sets forth results from one of those replications in which the leaf areas of leaves numbered 7, 8, 9, 10, 11, and 12, from Cxc inoculated plants, were significantly greater than their respective controls.

Table 3

Effect of Cxc Inoculation of Leaf Area[a] of Tobacco (C-319) Challenged with TMV (REP#1)

	Method of Inoculation	Leaf Number 5	6	7	8	9	10	11	12
Experimental	Stem Stab with Cxc	94	159	184	108	263	173	100	51
	Stem Inject with Cxc	90	140	162	165	272	174	117	49
Control	Stem Stab with Water	88	199	135	133	201	124	70	30
	Uninoculated	108	165	133	52	195	126	67	34
a = Leaf area in sq cm LSD =		16	29	26	24	38	29	21	13

Leaf fresh weight exhibited a similar pattern, as depicted in Table 4. Leaves 7, 8, 9, 10, 11 and 12 from Cxc-inoculated plants exhibited significantly greater fresh leaf weight than control plants.

Table 4

Effect of Cxc Inoculation on Leaf Fresh Weight[a] of Tobacco (C-319) CHallenged With TMV (REP#1)

	Method of Inoculation	Leaf Number 5	6	7	8	9	10	11	12
Experimental	Stem Stab with Cxc	4.5	6.8	6.1	4.0	10.7	7.2	4.3	2.9
	Stem Inject with Cxc	4.1	6.1	6.0	3.4	11.0	7.5	5.4	3.0
Control	Stem Stab with Water	3.9	7.0	4.0	1.6	7.2	6.0	3.4	1.9
	Uninoculated control	4.3	5.7	4.5	1.7	8.0	5.9	3.6	2.2
a = Leaf fresh weight in grams									
LSD =		0.7	0.9	1.0	0.8	1.5	1.14	0.9	0.3

Similarly, as depicted in Table 5, inoculated plants exhibited increased plant height and weight compared to controls.

Table 5

Effect of Cxc Inoculation on Plant Height and Weight of Tobacco Variety C-319 Challenged with TMV (REP#1)

	Method of Inoculation	Plant Height (cm)	Plant Weight (g)
Experimental	Stem Stab with Cxc	46	211
	Stem Inject with Cxc	43	189
Control	Stem Stab with Water	38	161
	Uninoculated Control	42	166
	LSD =	5.5	25

EXAMPLE 4

SYSTEMIC RESPONSE IN ENDOPHYTE-INOCULATED TOBACCO PLANTS FOLLOWING CHALLENGE WITH TMV

In the same manner as Example 3, in three field experiments, Cxc-inoculated plants exhibited significantly greater leaf area and leaf weight when challenged fourteen and twenty days post inoculation. As shown in Figures 1 and 2, the average leaf area of Cxc-inoculated plants was greater than control plants. This pattern was exhibited when challenge occurred at either fourteen days (Figure 1) or twenty days (Figure 2) after inoculation. Similarly, leaf weight of Cxc-inoculated plants challenged fourteen (Figure 3) or twenty days post inoculation (Figure 4) was significantly greater than that of control plants.

EXAMPLE 5

SYSTEMIC RESPONSE IN ENDOPHYTE-INOCULATED TOMATO PLANTS FOLLOWING CHALLENGE WITH TMV

Using the protocol of Example 1, tomato plants (Lycopersicon esculentum cv. Marglobe), susceptible to TMV, were planted in a greenhouse, transplanted into one gallon pots approximately six days later, and allowed to continue to grow in the greenhouse. This experiment was replicated twice. In both replications, plants were inoculated with Cxc one week after transplanting. All plants were challenged with TMV approximately three weeks after Cxc inoculation.

Approximately 25 days after challenge, the plants were assessed for the number of flowers produced, the number of fruiting bodies produced, and plant height. Table 6 sets forth the effect of Cxc inoculation on flowering and fruiting. Flowering scores were higher for inoculated plants than controls. Similarly, inoculated plants exhibited greater fruiting than did controls.

Table 6

Effect of Cxc on the Flowering and Fruiting of TMV Challenged Tomato

	TREATMENT	FLOWERING SCORE	FRUITING SCORE
Experimental	Stem stab with Cxc	2.3[a]	1.4[a]
	Stem inject with Cxc	1.9	1.2
Control	Stem stab with Water	1.5	1.0
	Stem inject with Water	1.8	1.0

a = Scores:
3=100% of plants flowering or fruiting
2=50 % of plants flowering or fruiting
1=0 % of plants flowering or fruiting

When TMV challenge of tomato plants was performed in the field, Cxc-inoculated plants exhibited an increase in yield over control plants, as set forth in Table 7.

Table 7

Effect of Cxc Inoculation on Yield of Tomato Plants Following Challenge with TMV

	Method of Inoculation	Tomato Yield (Kg)
Experimental	Stem Stab with Cxc	2.8
	Stem Inject with Cxc	3.3
Control	Stem Stab with Water	2.3
	Stem Inject with Water	2.7
	Unioculated Control	2.7
	LSD=	.6

Similarly, when the quality of tomatoes at harvest time was assessed, as set forth in Table 8, fruit from the Cxc-inoculated plants was of superior quality. In contrast, ratings for controls were predominantly in the lowest quality categories (three and four).

Table 8

Effect of Cxc Inoculation on Tomato Fruit Quality Following TMV Challenge with TMV

	Method of Inoculation	Quality Category[a] 1[b]	2	3	4	Average
Experimental	Stem Stab with Cxc	0%	12%	80%	8%	3.0
	Stem Inject with Cxc	0%	27%	68%	5%	2.7
Control	Stem Stab with Water	0%	0%	62%	38%	3.4
	Stem Inject with Water	0%	0%	59%	41%	3.4
	Uninoculated Control	0%	4%	48%	48%	3.4

a = % of total plants in each quality category
b = Quality categories:
1=mostly red
2=half red and half green
3=mostly green and large
4=mostly green but small

EXAMPLE 6

SYSTEMIC RESPONSE IN ENDOPHYTE-INOCULATED POTATO PLANTS FOLLOWING CHALLENGE WITH POTATO VIRUSES

In a field experiment, one hundred and eight potato plants (Solanum tuberosum cv. Kennebec) were planted by hand in a randomized complete block design with 3 replications.

After approximately seventeen days of growth, plants were treated with Cxc, introduced by either stem injection or stem stabbing, as described in Example 1 above. Control plants were stem injected with water, stem stabbed with water, or left untreated.

Twenty days after inoculation with Cxc, the potato plants were challenged with potato virus X (PVX) using the same procedure used for challenging tobacco plants with TMV as described in Example 1.

Plants were scored for flowering and for disease severity. As set forth in Table 9, potato plants inoculated by either injection or stabbing with Cxc exhibited a significantly higher percentage of flowering than did any of the three sets of control plants. Similarly, inoculated plants exhibited reduced disease severity (as evidenced by the number of discolored and wilted leaves) than controls. Disease severity in each plant was rated on a scale of 1-4 based on quality of leaves, such that:

1 = less than 10% of plants' leaves exhibited disease symptoms
2 = greater than 10% to less than 30% of the leaves exhibited disease symptoms
3 = greater than 30% but less than 60% of the leaves exhibited disease symptoms
4 = greater than 60% of the leaves exhibited disease symptoms

Table 9

Effect of Cxc Inoculation on Potato Flowering and Disease Severity Following Challenge with PVX

		DISEASE RATING[a]					FLOWERING[b]
	METHOD OF INOCULATION	1	2	3	4	AVERAGE DISEASE RATING	
Experimental	Stem stab with Cxc	4%	87%	9%	0%	1.9	52%
	Stem inject with Cxc	5%	76%	19%	0%	2.2	71%
Control	Stem stab with Water	0%	64%	32%	4%	2.3	20%
	Stem inject with Water	0%	52%	40%	8%	2.6	24%
	Uninoculated Control	0%	77%	14%	9%	2.3	9%

a = % of total plants in each disease rating
b = % of total plants flowering in each category

Example 7

SYSTEMIC RESPONSE IN ENDOPHYTE-INOCULATED POTATO PLANTS FOLLOWING CHALLENGE WITH POTATO VIRUSES

In the same manner as Example 6, 180 potato plants (S. tuberosum. cv. Kennebec) were planted by hand in the field. Approximately two weeks later, the plants were randomly assigned to five groups and subjected to five treatments. As above, the five treatments were: stem stab with Cxc, stem inject with Cxc, uninoculated control, stem stab with water and stem inject with water. Twenty days after inoculation with Cxc, all plants were challenged with potato virus Y (PVY)

Thirty days later, the plants were scored for flowering and for disease severity. As set forth in Table 10, although there was no difference in flowering between Cxc-inoculated and control plants, Cxc-inoculated plants did exhibit a reduction in disease severity over control

plants. Specifically, as set forth in Table 10, Cxc-inoculated plants predominantly ranked in the lowest damage category.

Table 10

Effect of Cxc Inoculation on Potato Flowering and Disease Severity Following Challenge with PVY

		DISEASE RATING[a]					FLOWERING[b]
	METHOD OF INOCULATION	1	2	3	4	AVERAGE DISEASE RATING	
Experi-mental	Stem stab with Cxc	22%	78%	0%	0%	1.8	65%
	Stem inject with Cxc	5%	73%	23%	0%	2.2	55%
Control	Stem stab with Water	0%	80%	20%	0%	2.2	48%
	Stem inject with Water	0%	50%	45%	5%	2.6	65%
	Uninoculated Control	0%	67%	29%	4%	2.4	46%

a = % of total plants in each disease rating
b = % of total plants flowering in each category

EXAMPLE 8

SYSTEMIC RESPONSE IN ENDOPHYTE-INOCULATED POTATO PLANTS FOLLOWING CHALLENGE WITH POTATO VIRUSES

In the same manner as Example 6, 180 potato plants were planted in the field and allowed to grow for approximately two weeks. The plants were then inoculated with Cxc, using the same five treatments as set forth above. Eighteen days after inoculation with Cxc, the plants were challenged with a mixture of PVY and PVX.

Seventeen days later the plants were evaluated. As set forth in Table 11, the Cxc-inoculated plants exhibited a greater percentage of flowering than did the control

plants. Similarly, Table 11 depicts that the Cxc-inoculated plants exhibited less damage due to viral infection than did the control plants.

Table 11

Effect of Endophyte-Inoculation on Potato Flowering and Disease Severity Following Challenge with PVX + PVY

		DISEASE RATING[a]					FLOWERING[b]
	METHOD OF INOCULATION	1	2	3	4	AVERAGE DISEASE RATING	
Experimental	Stem stab with Cxc	77%	23%	0%	0%	1.2	88%
	Stem inject with Cxc	77%	23%	0%	0%	1.2	95%
Control	Stem stab with Water	4%	84%	12%	0%	2.1	68%
	Stem inject with Water	12%	72%	16%	0%	2.0	80%
	Uninoculated Control	7%	86%	7%	0%	2.0	42%

a = % of total plants in each disease rating
b = % of total plants flowering in each category

EXAMPLE 9

SYSTEMIC RESPONSE OF ENDOPHYTE-INOCULATED TOMATO CHALLENGED WITH CLAVIBACTER MICHIGANENSE SUBSP. MICHIGANENSE

Using the protocol of Example, tomato plants (Lycopersicon esculentum cv. Marglobe) were planted in a greenhouse and allowed to grow for twelve days. Thereafter, plants were divided into five groups as follows:

1) untreated control
2) stem stab with water
3) stem inject with water
4) stem stab with Cxc
5) stem inject with Cxc.

Eleven days later, the tomato plants were challenged by introduction of the bacteria Clavibacter michiganense subsp. michiganense ("Cmm") (syn. Corynebacterium michiganense subsp. michiganese) grown on

nutrient broth yeast extract agar (NBY) and incubated at 26°C for approximately four days. Cell suspensions of Cmm in distilled water were used for challenge inoculation. Challenge inoculation was performed by breaking the petiole and applying a suspension containing approximately 10^8 cells/ml to the broken area.

Eighteen days after challenge, the plants were scored for disease severity. The severity of disease in each plant was rated on a scale of 0 to 4 as follows:

0 = no evidence of disease

1 = 0-10% of plant wilted

2 = 10%-40% of plant wilted

3 = 40-75% of plant wilted

4 = greater than 60% of plant wilted

As set forth in Table 12, the inoculated plants exhibited a dramatic reduction in disease compared to control plants.

Table 12

Effect of Endophyte-Inoculation on Tomato Disease Severity Following Challenge with C. michiganense subsp. michiganense

		DISEASE RATING				
	METHOD OF INOCULATION	1[a]	2	3	4	AVG.
Experimental	Stem stab with Cxc	33%	56%	11%	0%	1.8
	Stem inject with Cxc	22%	56%	22%	0%	1.9
Control	Stem stab with Water	0%	20%	60%	20%	3.0
	Stem inject with Water	0%	20%	80%	0%	2.8
	Uninoculated Control	0%	0%	100%	0%	3.0

a = % of total plants in each disease category

EXAMPLE 10

SYSTEMIC RESPONSE IN ENDOPHYTE-INOCULATED TOBACCO PLANTS FOLLOWING CHALLENGE WITH PSEUDOMONAS SYRINGAE pv. TABACI

Using the protocol of Example 1, tobacco plants were planted in the greenhouse. After an initial growth period of about 1 month, in each of three randomly assigned replications, ten plants were stem injected with Cxc, ten were stem stabbed with Cxc, five were stem stabbed with sterilized tap water, and five were untreated.

Fourteen days after inoculation with Cxc, the plants were challenged with Pseudomonas syringae pv. tabaci ("Ps. tabaci"). At 1, 2, 3 and 4 days after inoculation with this bacterium, a disc was punched from the leaf tissue between lesions. The discs were homogenized and plated onto Kings B Agar to calculate the number of Ps. tabaci cells per gram of leaf tissue. These numbers provided an index of bacterial infestion.

As set forth in Figure 5, inoculated plants exhibited a reduction in the number of bacterial cells per gram of leaf tissue each day after inoculation. Accordingly, it appeared that the Cxc-inoculated plants permitted less multiplication of Ps. tabaci in leaf tissue. This reduction of pathogen/titer in the plants has a direct impact on the rate of spread of the resultant disease (wildfire disease) in the field.

EXAMPLE 11

SYSTEMIC RESPONSE IN ENDOPHYTE-INOCULATED MUSKMELON CHALLENGE WITH FUSARIUM OXYSPORUM f.sp. MELONIS

Cucumis melo Muskmelon (variety Honey Rock) plants were grown in a greenhouse. At approximately one week post-emergence, plants were subjected to one of two treatments: hypodermal inoculation with washed Cxc cells resuspended in phosphate buffered saline ("PBS") at approximately 10^8 CFU/ml; or a control inoculation using PBS alone. After approximately 26 days of growth, all

plants were challenged with Fusarium oxysporum f.sp. melonis by transplanting into pots containing Fusarium infested soil. All plants were allowed to grow with a photoperiod of about 14:10 (L:D), at 95% relative humidity and 23°C until symptoms appeared. Two replications were conducted.

Plants were assessed for disease severity and plant dry weight. Plant weight was determined by weighing the harvested, above-ground portions of the plant. The severity of each disease was assessed using standard phytopathological methods and plants were rated on a scale of 0-5:

0 = no evidence of disease
1 = 0 - 10% of plant wilted
2 = 10% - 30% of plant wilted
3 = 30% - 60% of plant wilted
4 = greater than 60% of plant wilted
5 = plant death.

As set forth in Table 13, Cxc-inoculated plants exhibited greater dry weight and reduced disease severity compared to controls.

Table 13

Effect of Cxc Inoculation on Vascular Wilt of Muskmelon Following Challenge with Fusarium oxysporum f.sp. melonis.

Treatment	Mean Disease Severity Score	Mean Dry wt. (g)
Replication 1		
Cxc-inoculated	1.7	1.85
PBS-inoculated control	3.7	1.58
Replication 2		
Cxc-inoculated	2.58	4.01
PBS-inoculated control	3.75	2.15

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and the examples be considered as exemplary only, with the true scope of the spirit of the invention being indicated by the following claims and their equivalents.

WHAT IS CLAIMED IS:

1. A method of endophyte-enhanced protection in commercially-valuable plants comprising:

a. providing an endophytic organism that can be harbored within the plant and which creates no visible manifestations of disease, and

b. introducing the organisms to the plants such that the organisms enhance protection against disease.

2. The method of claim 1 wherein the endophytic organism is a vascular-inhabiting endophyte.

3. The method of claim 1 wherein the endophytic organism is a vascular-inhabiting bacterium.

4. The method of claim 2, wherein the vascular-inhabiting endophytic organism is a gram positive organism.

5. The method of claim 4 wherein the vascular-inhabiting gram positive endophytic organism is fastidious.

6. The method of claim 1 wherein the endophytic organism is a Coryneform bacterium.

7. The method of claim 1 wherein the endophytic organism is a Clavibacter spp.

8. The method of claim 1 wherein the endophytic organism is a Clavibacter xyli subsp. cynodontis.

9. The method of claim 1 wherein Clavibacter xyli subsp. cynodontis is introduced to tobacco plants to enhance protection against disease.

10. The method of claim 9 wherein the disease is caused by a virus.

11. The method of claim 10 wherein the virus is tobacco mosaic virus.

12. The method of claim 9 wherein the disease is caused by bacteria.

13. The method of claim 12 wherein the bacterium is Pseudomonas syringae pv. tabaci.

14. The method of claim 1 wherein Clavibacter xyli subsp. cynodontis is introduced to tomato plants to enhance protection against disease.

15. The method of claim 14 wherein the disease is caused by a virus.

16. The method of claim 15 wherein the virus is a tobacco mosaic virus.

17. The method of claim 14 wherein the disease is caused by a bacterium.

18. The method of claim 17 wherein the bacterium is Clavibacter michiganese subsp. michiganese.

19. The method of claim 1 wherein Clavibacter xyli subsp. cynodontis is introduced to potato plants to enhance protection against disease.

20. The method of claim 19 wherein the disease is caused by virus.

21. The method of claim 20 wherein the disease is caused by potato virus X.

22. The method of claim 20 wherein the disease is caused by potato virus Y.

23. The method of claim 20 wherein the disease is caused by a combination of potato virus X and potato virus Y.

24. The method of claim 1 wherein Clavibacter xyli subsp. cynodontis is introduced to muskmelon to enhance protection against disease.

25. The method of claim 24 wherein the disease is caused by a fungus.

26. The method of claim 24 wherein the disease is caused by vascular wilt fungus.

27. The method of claim 26 wherein the fungus is a Fusarium sp.

28. The method of claim 1 wherein the endophytic organism is genetically unmodified.

29. The method of claim 1 wherein the endophytic organism is genetically modified.

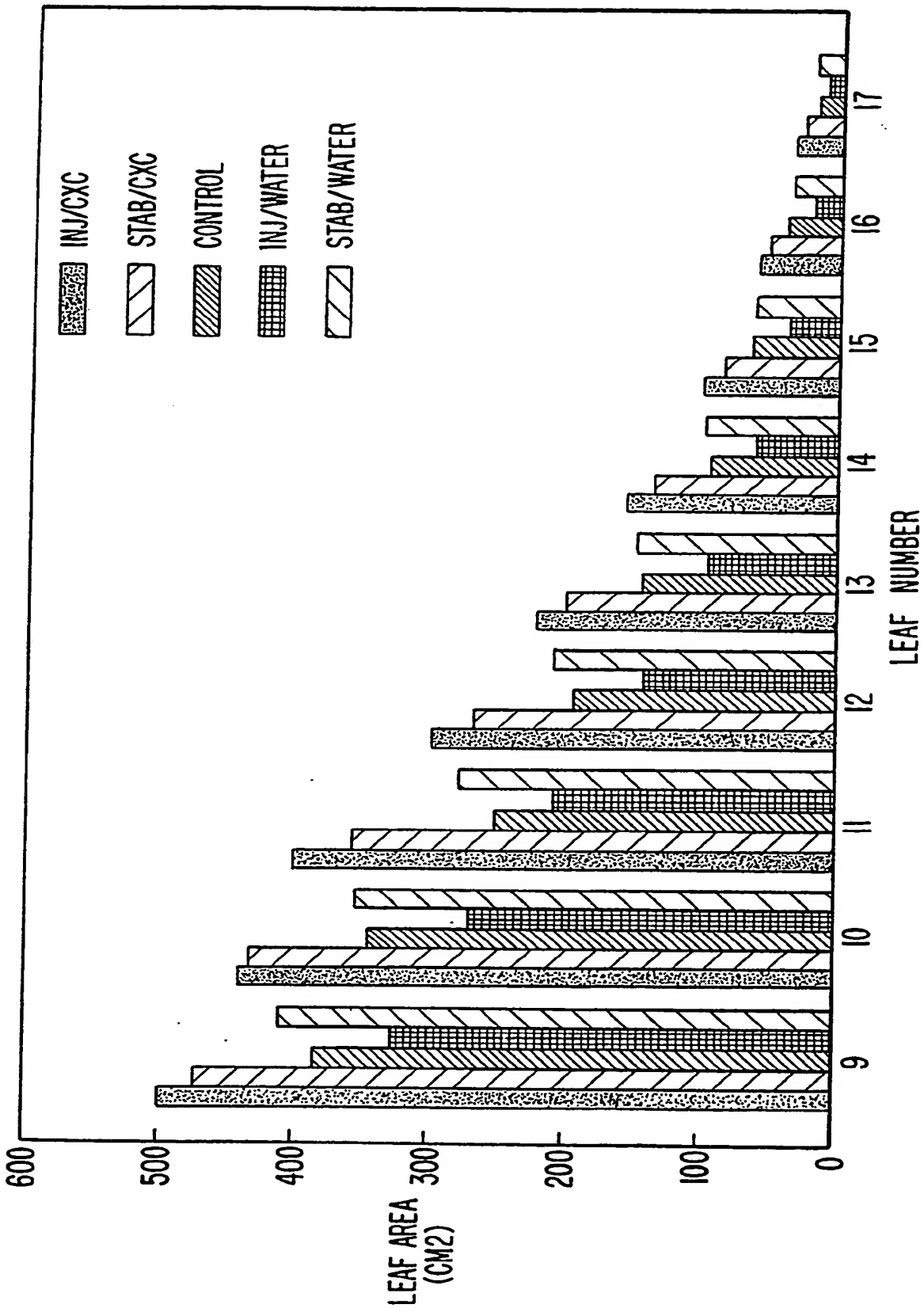
30. The method of claim 28 wherein the endophytic organism is modified by recombinant DNA techniques.

31. The method of claim 28 wherein the endophytic organism is modified by mutagenesis techniques.

32. The method of claim 1 wherein the endophytic organism is formulated with one or more abiotic inducers.

33. The method of claim 1 wherein the endophytic organism creates no visible manifestation of disease.

FIG. 1.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STAB/CXC
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INJ/WATER
STAB/WATER
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10
11
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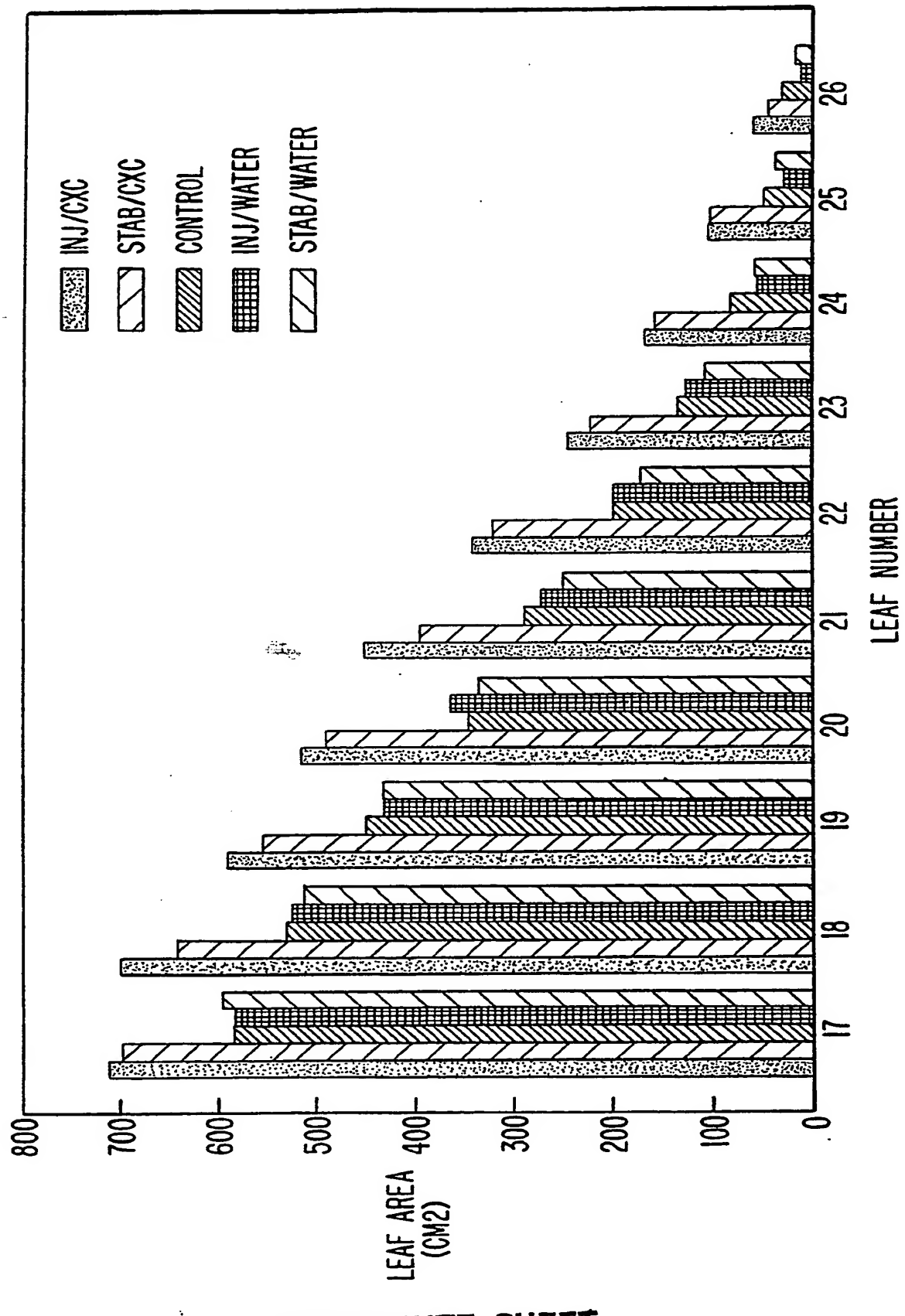
16
17
LEAF NUMBER

FIG. 2.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STAB/CXC
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INJ/WATER
STAB/WATER
800
700
600
500
400
300
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17
18
19
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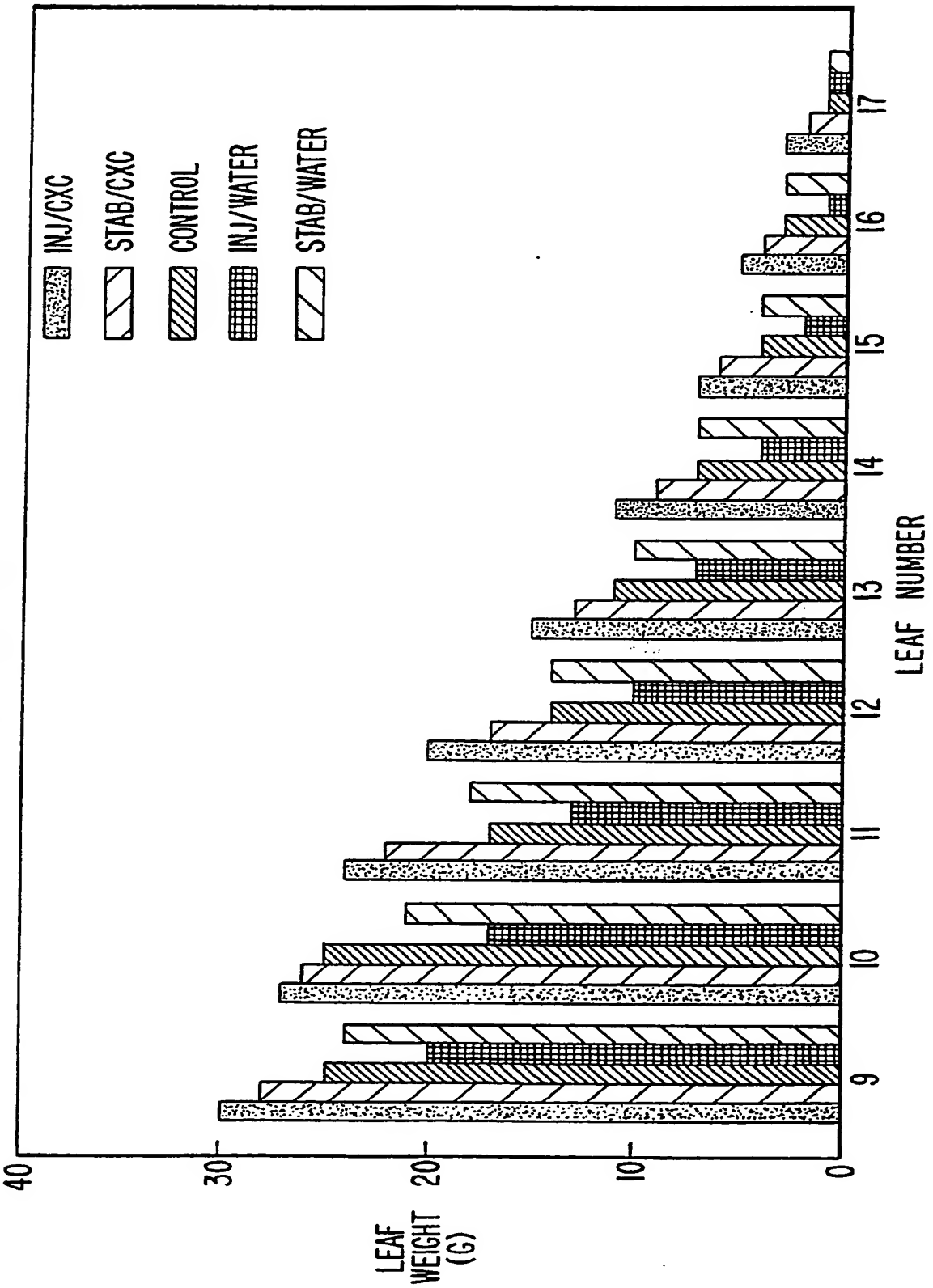
LEAF NUMBER

FIG. 3.


INJ/CXC
STAB/CXC
CONTROL
INJ/WATER
STAB/WATER
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40
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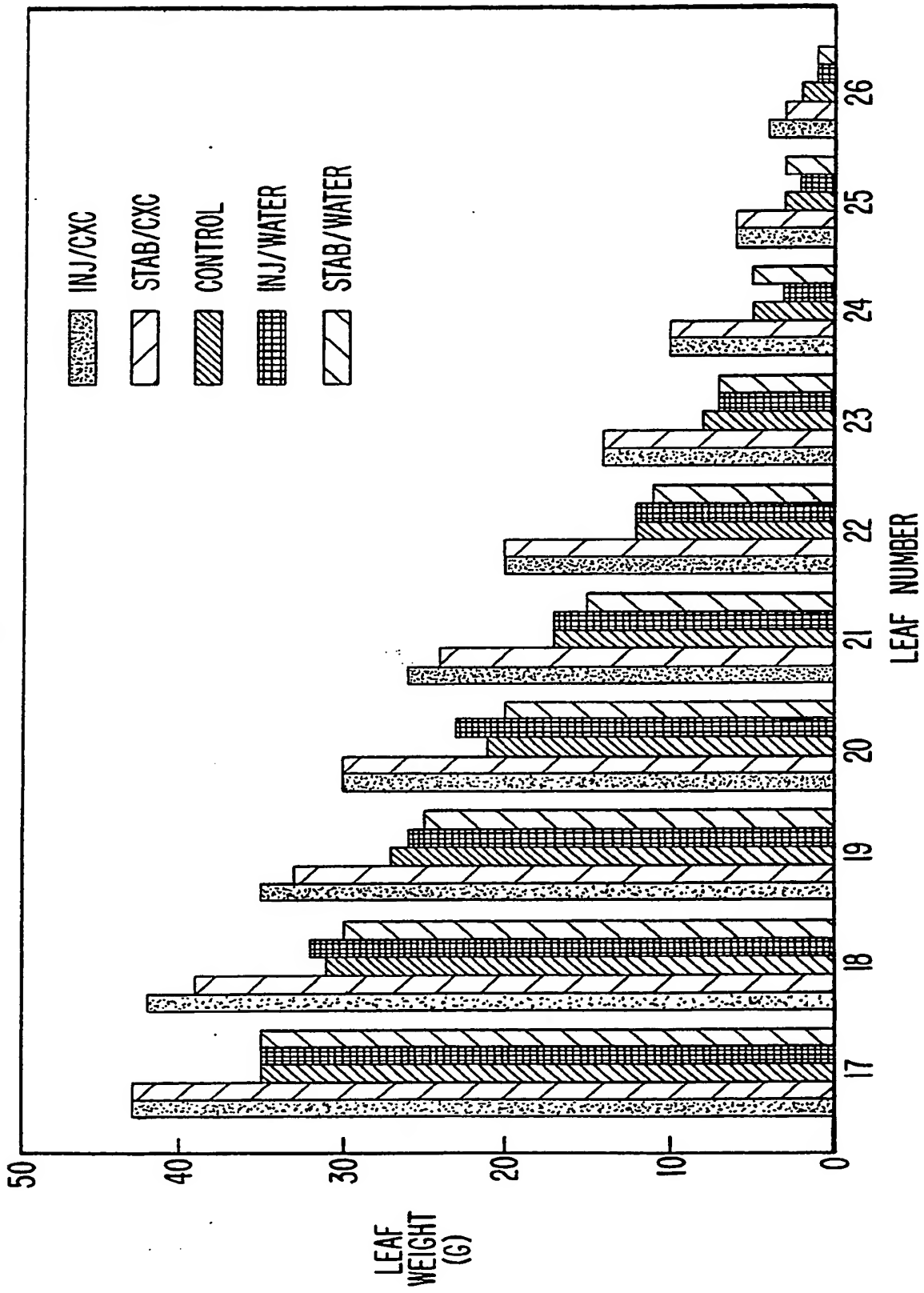
10
11
12
13
14
15
16
17
LEAF NUMBER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STAB/CXC
CONTROL
INJ/WATER
STAB/WATER
LEAF WEIGHT (G)
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50
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21
22
23
24
25
26
LEAF NUMBER

FIG. 4.

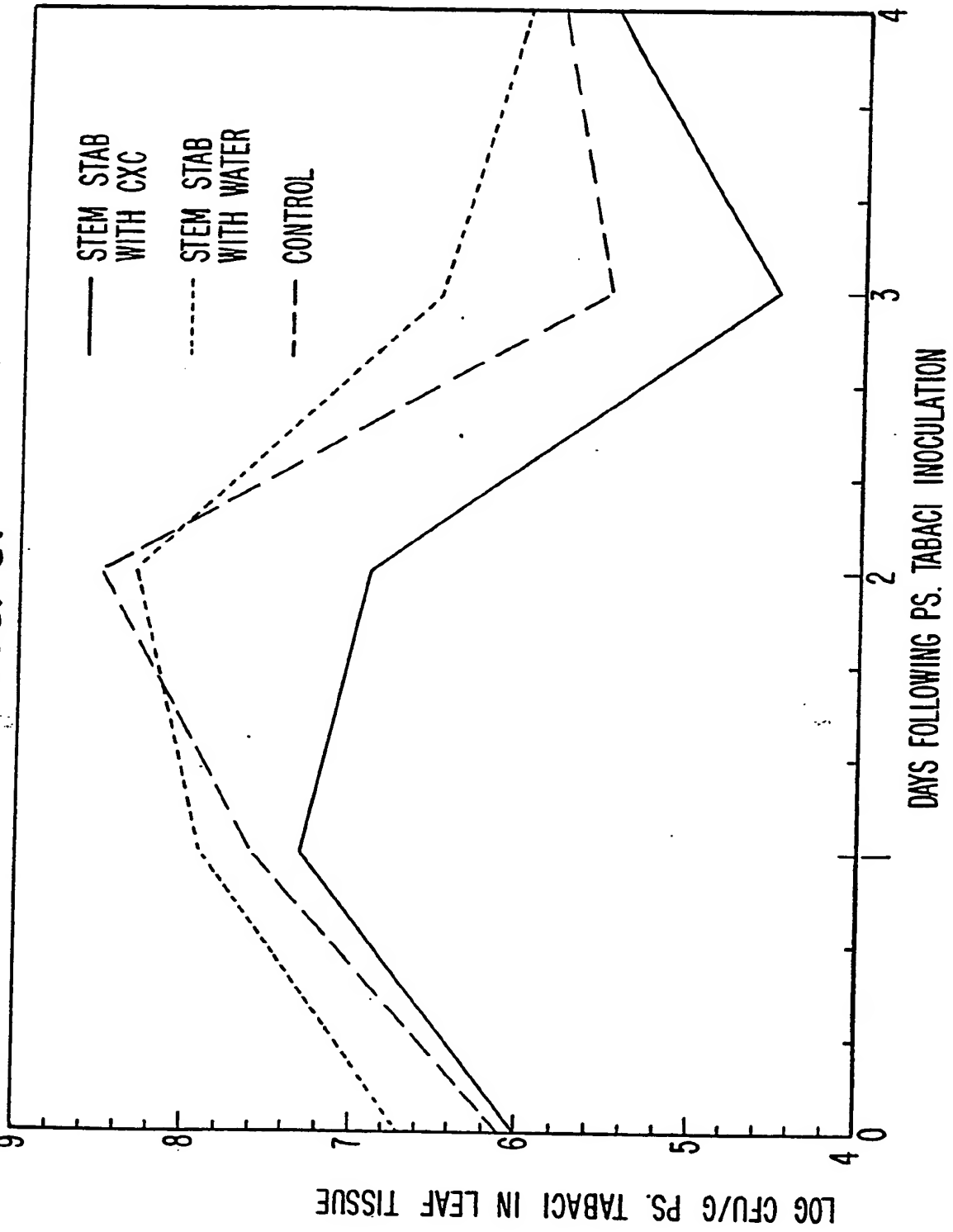
STEM STAB WITH CXC
STEM STAB WITH WATER
CONTROL
LOG CFU/G PS. TABACI IN LEAF TISSUE
DAYS FOLLOWING PS. TABACI INOCULATION
9
8
7
6
5
4
0
1
2
3
4

FIG. 5.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02240

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A01N 63/00; C12N 1/00, 1/14, 1/20
US CL: 424/93, 435/252.3, 252.34, 252.32, 254

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/93; 435/252.3, 252.34, 252.32, 843, 929, 254

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

DATABASES: Chemical abstracts on line (File Biosis, 1969-1990); USPTO Automated Patent System (File USPTO, 1975-1990). See attachment for Search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	Phytopathology (St. Paul, MN, U.S.A.), volume 78, No. 12, issued 1988, Brooks, et al, "Evaluation of endophytiz Pseudomonas spp. isolated form live Oak Trees for potentia biological control of Oak wilt, See abstract.	1, 28 1-33
Y	Forest Science (Bethesda, MD), volume 24, No. 4, issued 1978, Leben, "Biological Control of decay fungi; a wood disk evaluation method", pages 560-564, see the entire document.	1-33
Y	Physiological Plant Pathology (London, England) volume 16, issued 1980, Sziraki, et al, "Role of different stresses in inducing systemic acquired resistance to TMV and increasing cytokinin level in tobacco," pages 277-284, see the entire document	1-33
Y	Annual review of Phytopathology (Palo Alto, CA), volume 24, issued 1986, Davis, "Taxonomy of Plant-pathogenic coryneform bacteria," see pages 115-116 and 130-135.	1-33

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
05 JUNE 1990	09 AUG 1990
International Searching Authority [1]	**Signature of Authorized Officer**
ISA/US	NGUYEN NGOC HA INTERNATIONAL DIVISION BARBARA M. CHERESKIN

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	BioScience (Washington, D.C.) volume 32, No. 11, issued 1982, Kuc, "Induced immunity to plant disease" pages 854-860, see the entire document	1-33
Y	Netherlands Journal of Plant Pathology (Wagen ingen, The Netherlands), volume 89, issued 1983, Van Loon, "the induction of pathogenesis-related proteins by pathogens and specific chemicals", pages 265-273, See especially Table 1.	1-33
Y	Applied and Environmental Microbiology (Washington, D.C.), volume 53, No. 12, issued December 1987, Juhnke et al. "Identification and characterization	1-33

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ...., because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
	of rhizosphere-competent bacteria of wheat", pages 2793-2799, see the entire document.	
A	Trends in Biotechnology (Amsterdam, Netherlands), volume 7, issued February 1989, Kloepper et al, "Free-living bacterial inocula for enhancing crop productivity' pages 39-44, see the entire document.	1-33
X	Journal of Chemical Ecology (New York, NY), volume 12, issued 1986, Rowan et al. "Isolation of feeding deterrents against argentine stem weevil from rye grass infected with the endophyte Acremonium loliae, "pages 647-658, see the entire document.	1-33
Y	Plant Disease (St. Paul, MN), volume 71, No. 9, issued September 1987, Redmond et al, "Biological Control of Botrytis Cinerea on roses with epiphytic microorganisms", pages 799-802, see the entire document.	1-33
Y	Journal of the American Society of Horticultural Scientists (St. Joseph, MI, USA), volume 114(1), issued January 1989, Hammer et al, "Nonchemical methods for postharvest control of Botrytis Cinerea on cut roses", pages 100-106, see the entire document.	1-33